

Selection of Tumor Antigens as Targets for Immune Attack Using Immunohistochemistry: Protein Antigens^{1,2}

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ABSTRACT

The relative expression of mucin antigens MUC1, MUC2, MUC3, MUC4, MUC5_{AC}, MUC5_R, and MUC7 and glycoprotein antigens KSA, carcinoembryonic antigen, prostate-specific membrane antigen (PSMA), HER-2/*neu*, and human chorionic gonadotropin- β on different cancers and normal tissues is difficult to determine from available reports. We have compared the distribution of these antigens by immunohistology on a broad range of malignant and normal tissues. MUC1 expression was most intense in cancers of breast, lung, ovarian, and endometrial origin; MUC2 was most intense in cancers of colon and prostate origin; and MUC5_{AC} was most intense in cancers of breast and gastric origin. MUC4 was intensely expressed in 50% of cancers of colon and pancreas origin, and MUC3, MUC5_R, and MUC7 were expressed in a variety of epithelial cancers, but not so intensely. KSA was intensely and uniformly expressed on all epithelial cancers; carcinoembryonic antigen was expressed in most cancers of breast, lung, colon, pancreas, and gastric origin; and PSMA was expressed only in cancers of prostate origin. Human chorionic gonadotropin- β was expressed on the majority of sarcomas and cancers of breast, lung, and pancreas origin, although intense staining was not seen. Staining on normal tissues was restricted to one or many normal epithelial tissues ranging from MUC3, MUC4, and PSMA, which were expressed only on epithelia of pancreas, stomach, and prostate origin, respectively, to MUC1 and KSA, which were expressed on most normal epithelia. Expression was restricted to the secretory borders of these epithelia while stroma and other normal tissues were completely negative. These results plus the results of the two previous papers (S. Zhang *et al.*, *Int. J. Cancer*, 73: 42-49,

1997; S. Zhang *et al.*, *Int. J. Cancer*, 73: 50-56, 1997) in this series provide the basis for selection of multiple cell surface antigens as targets for antibody-mediated attack against these cancers.

INTRODUCTION

This is our third and final immunohistochemistry study comparing the expression of a series of cell surface antigens (selected as potential targets for immunotherapy) on a range of normal and malignant tissues. The previous two studies (1, 2) focused on carbohydrate epitopes expressed in glycolipids, mucins, and other glycoproteins. Here, we focus on the peptide epitopes of seven mucins and five glycoproteins, each of which is available for vaccine construction as a consequence of simple peptide synthesis (MUC1-MUC7) or expression in *Escherichia coli* or baculovirus (3-7). Each of these antigens is either known to be expressed at the cell surface as a consequence of a demonstrated transmembrane domain (MUC1, KSA, CEA,⁴ PSMA, and HER-2/*neu*; Refs. 8-12) or is thought to be shed by tumor cells and be either adherent to or abundant in the vicinity of tumor cells (MUC2, MUC3, MUC4, MUC5_{AC}, MUC5_R, and MUC7 and β hCG; Refs. 13-19). Although the expression of each of these antigens on human tumors and normal tissues has been described, previous studies were limited in terms of number and types of tissues studied, involved mAbs against only one to three antigens without direct comparison to expression of other antigens, and used different immunostaining procedures (indirect immunofluorescence, indirect immunoperoxidase, or ABC immunoperoxidase; Refs. 13 and 19-29). Consequently, the comparative distribution of these antigens on cancers and normal tissues is difficult to determine from available reports, although this is precisely the information required for selecting target antigens for immunotherapy. This is especially important with the recent development of conjugate vaccines capable of inducing antibodies in most patients against a variety of well-defined tumor antigens (30) and with the recent evidence that the induction of these antibodies correlates with a more favorable prognosis (30-32).

MATERIALS AND METHODS

Tissue Samples. Frozen specimens embedded in Tissue-Tek O.C.T. compound (Diagnostic Division, Elkhart, IN) were provided with pathological reports by the Tissue Procurement Service of Memorial Sloan-Kettering Cancer Center (New York, NY), with the exception of four frozen specimens of metastatic prostate cancer, which were kindly provided by Dr.

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¹ This paper is the third in a series. See Refs. 1 and 2.

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⁴ The abbreviations used are: CEA, carcinoembryonic antigen; β hCG, human chorionic gonadotropin- β ; mAb, monoclonal antibody; ABC, avidin-biotin complex; GI, gastrointestinal; PSMA, prostate-specific membrane antigen.

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Table 1 Mouse mAbs used for immunohistology

mAb	Ig class	Antigen	Antigen structure	Ref.
HMFG-2	IgG1	MUC1	VTSPDTRPAPGSTAPPAHG repeating	41, 42
LDQ10	IgM	MUC2	PTTTPISTTTTVPPTPTPTGTQT repeating	37
M3.2	IgG2a	MUC3	HSTPSFTSSITTTETTS repeating	20
MUC4.275	IgG1	MUC4	TSSASTGHATPLPVTG repeating	43
CLH2	IgG1	MUC5 _{ac}	TTSTTSAP repeating (interrupted)	27
PANH2	IgG1	MUC5 _n	No peptide repeats	44, 45
PANH3	IgG1	MUC7	TTAAPPTPSATTAPPSSSAPPE repeating	44, 45
NCL-CEA	IgG1	CEA	Glycoprotein (M 180,000)	Vector Laboratories
Cyt351	IgG	PSMA	Glycoprotein (M 100,000)	28, 46
GA733-2	IgG2a	KSA	Glycoprotein (M 40,000)	47
FB12	IgG1	βhCG	145-amino acid glycoprotein	48
NCL-CBE1	IgG2a	HERv2/neu	Protein (M 185,000)	8

Table 2 Proportion of cancer specimens with ≥50% positive cancer cells (≥2+ staining intensity) by immunohistology^a

Cancer	Antigen (mAb)										HER-2/neu	
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (M3.2)	MUC4 (M4.275)	MUC5 _{ac} (CLH2)	MUC5 _n (PANH2)	MUC7 (PANH3)	KSA (GA733-2)	PSMA (Cyt351)	CEA (NCL-CEA)	βhCG (FB12)	(NCL-CBE1)
Melanoma	0/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Sarcoma	0/5	0	0	0	0	1/5	0	0	0	0	3/5	0
Neuroblastoma	0	1/5	0	0	0	0	0	0	0	0	1/5	0
B-cell lymphoma	0/5	0	0	0	0	0	0	0	0	0	0	0
Small cell lung	1/5	0	0	0	0	0	0	5/5 ^b	0	3/5	1/5	0
Breast	5/7 ^b	3/7	1/7	1/7	5/6 ^b	4/6	1/6	5/7 ^b	0/7	4/7 ^b	4/7	1/7
Metastatic prostate	3/5	4/5 ^b	0	0	1/5	0	0	5/5 ^b	3/5 ^b	0	1/5	1/5
Lung	4/5 ^b	0	1/5	4/5	0	0	0	5/5 ^b	0	4/5 ^b	3/5	1/5
Colon	3/8	6/8 ^b	4/8	4/8 ^b	1/8	3/8	4/8	8/8 ^b	0	6/8 ^b	2/8	0/8
Pancreas	2/5	2/5	0	3/5 ^b	2/5	1/5	0	5/5 ^b	0	3/5 ^b	4/5	0
Gastric	1/5	0	3/5	2/5	4/5 ^b	1/5	1/5	5/5 ^b	0	5/5 ^b	0	0
Ovarian	5/5 ^b	2/5	4/5	3/5	0	0	0	5/5 ^b	0	2/5	2/5	0
Endometrial	3/5 ^b	0	2/5	0	1/5	2/5	0	5/5 ^b	0	1/5	2/5	0

^a All tumor tissues were stained by ABC immunoperoxidase methods.^b Median staining intensity was 4+ for ≥80% of cells.

G. Steven Bova (PELICAN Laboratory, Johns Hopkins University, Baltimore, MD). Cryostat sections were cut at 5 μm, dried in air, and fixed with neutral buffered 10% formalin solution (Sigma Co., St. Louis, MO) for 10 min before H&E or immunostaining.

mAb and Immunohistochemistry. The murine mAbs and the antigens they recognize are summarized in Table 1. mAb HMFG-2 was provided by J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom); LDQ10 was provided by F. X. Real (Institut Municipal d'Investigació Mèdica, IMIM, Barcelona, Spain); M3.2 and MUC4.275 were provided by V. Apostolopoulos (Austin Research Institute, Victoria, Australia); CLH2, PANH2, and PANH3 were provided by H. Clausen (University of Copenhagen, Copenhagen, Denmark); Cyt351 was provided by W. Heston (Memorial Sloan-Kettering Cancer Center); FB12 was provided by D. Bellef (Institut Gustave-Roussy, Villejuif, France); and GA733-2 was provided by D. Herlyn (The Wistar Institute, Philadelphia, PA). mAbs NCL-CEA and NCL-CBE1 were purchased from Vector Laboratories, Inc. (Burlingame, CA).

The ABC immunoperoxidase method was performed as

described previously (33). Briefly, the sections were quenched with 0.1% H₂O₂ in PBS for 15 min, blocked with avidin and biotin reagents (Vector Laboratories) for 10 min each, incubated in 10% serum of horse or goat from which the second antibody was raised, and incubated with various mAbs for 1 h at optimal concentration. The optimal mAb concentration was selected based on the strongest reactivity against the known positive target cells with little or no background against stroma. The concentrations of mAbs used were: FB12, 0.5 μg/ml; Cyt351 and GA733-2, 2 μg/ml; HMFG-2, M3.2, MUC4.275, CLH2, PANH2, and PANH3 (supernatants), between 1:3 and 1:6; LDQ10 and NCL-CBE1 (ascites), 1:15; and NCL-CEA, 1:50. The sections were subsequently incubated with 1:600 biotinylated horse antimouse IgG or 1:300 goat antimouse IgM antibodies (Vector Laboratories) for 40 min and then incubated in 1:50 ABC reagent (Vector Laboratories) for 30 min. Reactions were developed with 0.02% H₂O₂ and 0.1% diaminobenzidine tetrahydrochloride (Sigma) for 2–5 min. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 1–3 min. The immunoreactivities were graded based on the percentage of positive cells and staining intensity above that seen on the negative control: 1+ (weak)

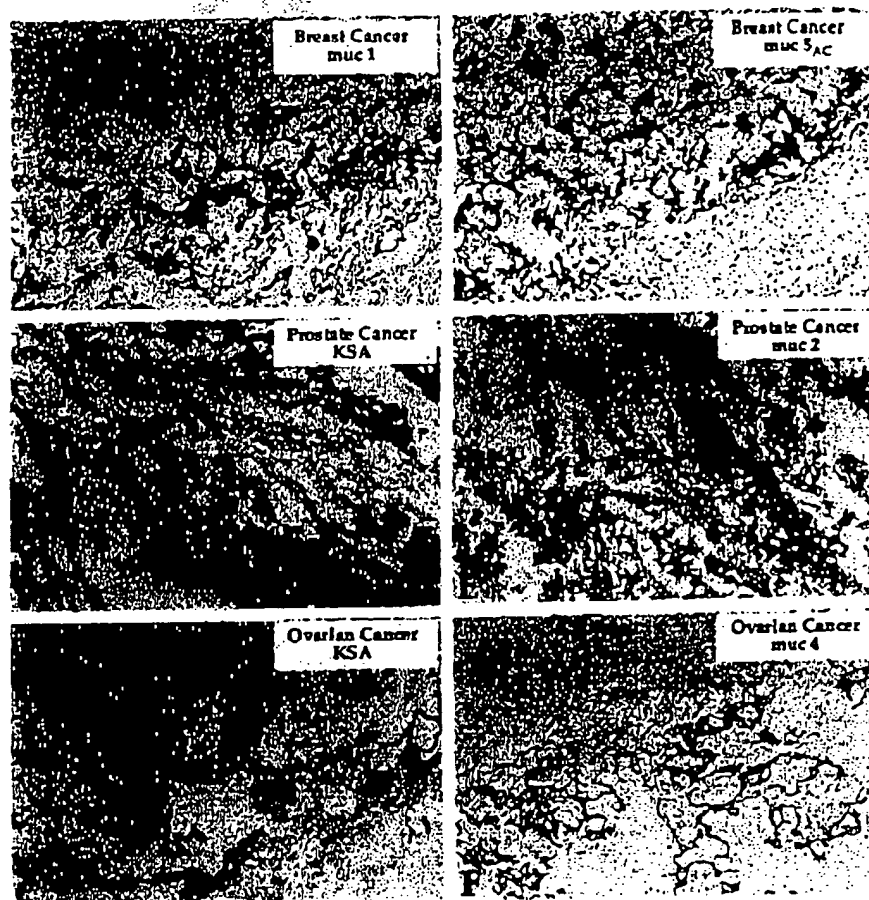


Fig. 1. Expression of protein antigens on breast cancer (A and D), prostate cancer (B and E), and ovarian cancer (C and F). The pattern of staining of cell membrane bound antigens MUC1 (A) and KSA (B and C) is indistinguishable from secreted antigens MUC5AC (D), MUC2 (E), and MUC4 (F). Staining intensity in these sections is graded as follows: A, 2-3+, 80% of tumor cells positive; B, 4+, 100%; C, 4+, 100%; D, 3+, 80%; E, 3+, 80%; F, 3+, 80%. Magnification, $\times 70$.

2+ (moderate), 3+ (strong), and 4+ (very strong or intense). Staining intensities of 2+ or stronger were considered positive (Table 2 and Fig. 1). Known positive and negative control slides were used in each experiment. Results with the several IgM, IgG3, and IgG2 mAbs included in the panel of antibodies tested ruled out nonspecific adherence of particular subclasses of antibodies.

An indirect immunoperoxidase assay was performed, as described previously (34) on normal liver, kidney, and stomach samples because these tissues reacted strongly with ABC reagent directly, producing high background. Briefly, the sections were quenched with 0.1% H_2O_2 in PBS for 15 min, blocked with 10% serum, and incubated with mAbs for 1 h at the optimal concentration. The sections were incubated with 1:100 rabbit antimouse immunoglobulin labeled with peroxidase (DAKO Corp., Carpinteria, CA) for 1 h and developed as described for the ABC method.

RESULTS

Reactivity of mAbs with Tumor Tissues. Table 2 summarizes the staining on tumor tissue samples observed with the panel of mAbs. Eighty-two neoplastic tissue specimens representing 13 tumor types were analyzed with each of the 12 antibodies. None of these mAbs reacted consistently with melanoma, neuroblastoma, or B-cell lymphoma specimens, and

only PB12 against βhCG reacted moderately (2+) with some sarcomas. KSA was very strongly expressed (median 4+) on small cell lung cancer and all or most specimens of all of the epithelial cancers. At the other extreme was PSMA, expressed only on primary and metastatic prostate cancer (median, 3+–4+). βhCG was expressed moderately (median, 2+) on some samples of most tumor types, but strong expression (3+) on occasional specimens, such as three of five lung cancer specimens, was also seen. CEA, MUC1, MUC2, and MUC4 were strongly expressed on the majority of some epithelial cancers (median, 3+–4+) but not expressed at all on others. MUC3, MUC5B, and MUC7 were moderately expressed on the majority of several cancers (median, 2+). MUC5AC was strongly expressed on only breast and gastric cancers. Confidence in all of these results was bolstered by the very strong expression (4+) seen on some specimens with each of these mAbs and complete lack of staining on other specimens. Strong (3+) HER-2/*neu* expression was only seen on one prostate cancer specimen, and the other two positive specimens were 2+, despite using the available ascites at a 1:15 dilution. Consequently, in the absence of a clear positive control, it is not clear whether the lack of staining of more specimens with NCL-CBE1 against HER-2/*neu* was a consequence of low levels of antigen expression, inactive antibody, or problems with the assay. Representative examples of these reactions and our grading

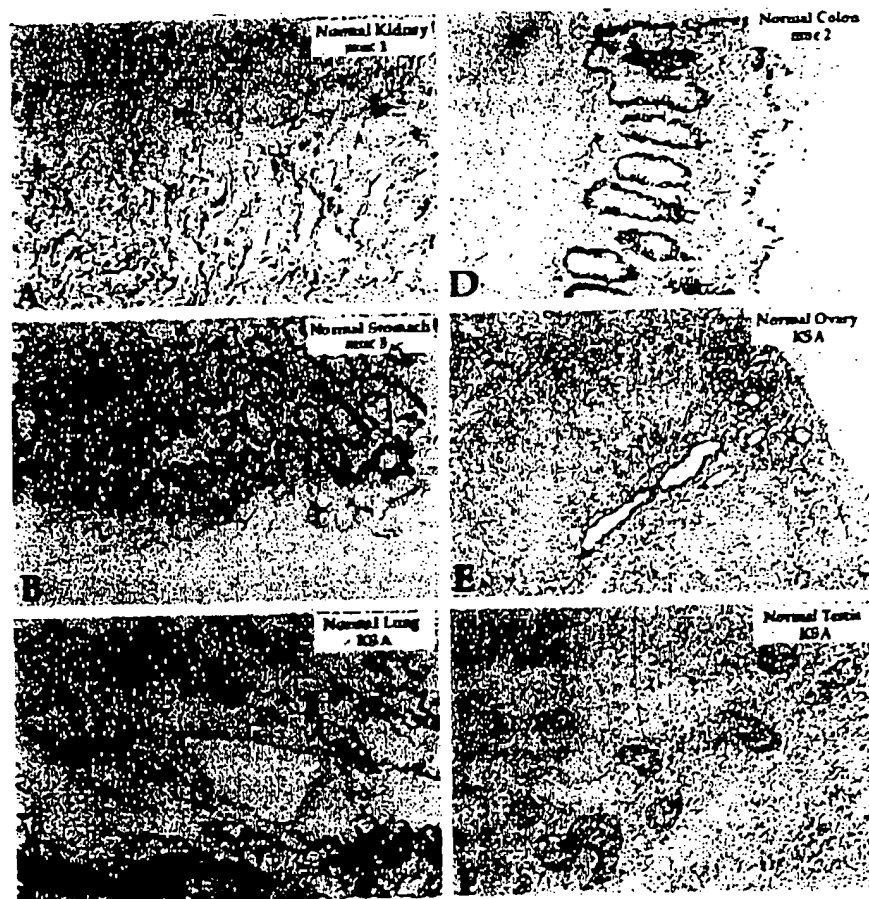
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Table 3 Antigen expression on normal tissues defined by immunohistoLOGY^a

Normal tissue (no.) ^b	Antigen (nAb)										
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (M3-2)	MUC4 (M4-275)	MUC5 _{ac} (CLH2)	MUC5 _a (PANTH2)	MUC7 (PANTH3)	KSA (GA733-2)	PSMA (Cy351)	CEA (NCL-CEA)	HER-2/neu (NCL-CEB1)
Spleen (2)	-	-	-	-	-	-	-	-	-	-	-
White pulp	-	-	-	-	-	-	-	-	-	-	-
Red pulp	-	-	-	-	-	-	-	-	-	-	-
Skeletal muscle (2)	-	-	-	-	-	-	-	-	-	-	-
Epithelia											
Lung (2)	2+	-	-	-	-	-	-	3+	-	1+	1+
Breast (2)	1+	-	-	-	-	-	-	3+	-	2+	2+
Prostate (6)	2+	2+	-	-	-	-	-	4+	3+	3+	-
Colon (2)	2+	3+	-	3+	-	3+	1+	4+	1+	1+	-
Stomach (2)	1+	-	-	-	4+	-	1+	-	-	2+	-
Pancreas (2)	2+	2+	2+	-	-	-	1+	4+	-	2+	-
Uterus (2)	1+	-	-	-	-	-	-	3+	-	1+	-
Ovary (2)	1+	-	-	-	-	-	-	3+	-	-	-
Liver (2)	-	-	-	-	-	-	-	-	-	-	-
Kidney (2)	2+	-	-	-	-	1+	-	1+	-	-	-
Testis (2)	-	-	-	-	-	1+	-	2+	-	2+	-
Tissues negative for all 12 antigens											
Brain (3): gray matter, white matter											
Lymph nodes (2)											
Smooth muscle (2)											
Connective tissue (2 each): lung, breast, prostate, colon, stomach, pancreas, uterus, ovary, liver, and kidney											

^a All tissues were stained by ABC immunoperoxidase method, except stomach, liver, and kidney, which were stained by the indirect immunoperoxidase method.^b The numbers in parentheses indicate the number of different specimens tested.^c Histiocytes in the red pulp were predominantly stained.^d Seminiferous tubules were stained.

Fig. 2 Expression of protein antigens on normal tissues. Epithelial cells at secretory borders were stained in kidney (A) with mAb HMPG2 against MUC1 (2+); in stomach (B) with mAb CLH2 against MUC5_{AC} (4+); in lung (C), ovary (E), and testis (F) with mAb GA733-2 against KSA (4+, 2+, and 3+, respectively) and in colon (D) with mAb LDQ10 against MUC2 (3+). Magnification, $\times 70$.



of percentage positive tumor cells and staining intensity are shown in Fig. 1. Staining of stroma with all 12 of these mAbs was uniformly negative.

Reactivity of mAbs with Normal Tissues. Table 3 summarizes the immunoreactivity on normal tissue samples observed with the panel of mAbs. MUC1 was weakly distributed on the epithelia of all of the tested organs, except liver. MUC2 was observed on the epithelia of prostate, colon, and pancreas. MUC3 was only detected on epithelia of pancreas. MUC4 was expressed on epithelia of colon and prostate (weakly). MUC5_{AC} was very strongly expressed in stomach epithelium. MUC7 and HER-2/*neu* were not expressed on any normal tissues, and MUC5_B was only detected on normal colon epithelium and weakly in the testis. β hCG was detected in epithelia of prostate, stomach, and pancreas and weakly in colon and lung, and it was detected in the testis. PSMA was only detected on prostate epithelia. KSA was strongly expressed on the epithelia of all of the tested organs except stomach and liver and moderately expressed in the epithelia of prostate and colon and weakly in lung, uterus and breast. The pattern of expression of each of these antigens on normal epithelia was mainly luminal, with evident polarity. Luminal cells stained most intensively at luminal borders. In addition, CEA was detected in histiocytes in the red pulp of the spleen, an expected consequence not of CEA

expression but of the mAb used, NCL-CEA, which cross-reacts with nonspecific cross-reacting antigen on histiocytes (35). Examples of the staining patterns on normal tissues with these mAbs are shown in Fig. 2. Once again, staining of stroma was uniformly negative.

DISCUSSION

One of the striking features of our two previous reports was the clear separation between the carbohydrate antigens expressed by tumors of neuroectodermal origin and the carbohydrate antigens expressed by tumors of epithelial origin (1, 2). This is also the case for the protein antigens studied here. None of the seven mucins were expressed on more than one of the specimens of the five nonepithelial origin cancers, but these mucins were widely and densely expressed on a variety of epithelial cancers. The same applies for the other glycoproteins, except that all small cell lung cancers expressed KSA very strongly and some expressed CEA, and some sarcomas expressed moderate amounts of β hCG. On this basis, melanomas, sarcomas, neuroblastomas, and B-cell lymphomas are quite distinct from the eight epithelial cancers tested. Small cell lung cancer, not surprisingly, is intermediary, with some characteristics of each group.

This study differs from previous reports on the distribution

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of these antigens in several ways. Our focus was entirely on potential targets for immunotherapy and, especially, on antibody-mediated immunotherapy. We have compared the expression of 12 antigens rather than 1 to 3 and explored a wide variety of malignant and normal tissues rather than a few. On the other hand, we tested only five to eight specimens in most cases, and because this was part of a larger study looking at expression of glycolipid antigens as well, specimens were fixed with 10% formalin, which may not be ideal for some protein antigens. However, to the extent that others have studied the expression of these antigens on these cancers, our study is largely in agreement. MUC1 has long been known to be expressed by many normal epithelial tissues and by many or most cancers of breast, ovary, pancreas, prostate, and colon origin (21-23, 26). We concur and add to this list endometrial and non-small cell lung cancer. MUC2 has been previously identified in most colon cancers as well as cancers of the stomach, pancreas, breast, and, recently, prostate (22, 24, 36). We concur, except that we found no evidence of MUC2 in the five gastric cancer specimens we tested. The previously described more restricted expression of MUC2 than MUC1 on normal tissues (22, 24, 36, 37), with MUC2 detected in the GI tract and, recently, the prostate (36) but not most other sites, was also our finding. MUC3 was previously detected on the majority of colon, ovarian, and gastric cancers (20, 24), in agreement with our findings, but also in the GI tract but not the pancreas, which is the reverse of our findings. MUC5_{AC} has previously been detected in the majority of gastric cancers and in normal stomach, as we found, and we add to this strong expression in most breast cancers (22, 27). MUC5_R has been described to be strongly expressed on some colorectal carcinomas and normal colon (22), as we found. We add to this moderate expression of MUC5_R on the majority of breast cancers. Our study breaks little new ground on the distribution of KSA (38) and PSMA (4, 28, 36), except that we were not prepared for the intensity and uniformity of KSA expression on all epithelial cancers tested (and normal epithelial tissues), and we have extended the number of different normal tissues and nonprostate cancers that are negative for PSMA by immunohistology. Likewise, we confirm the strong expression of CEA on most breast, lung, and GI malignancies and the corresponding normal tissues as described previously (35, 39, 40). β hCG mRNA has been described to be strongly expressed in 61% of bladder cancers (which we did not test) and to be moderately expressed in 46% of breast cancers and 20% of prostate cancers (19), which agrees with our findings. We add to this moderate expression in a small proportion of several other cancers and the majority of sarcomas and cancers of the lung and pancreas, as well as a variety of normal tissues.

A benefit of testing many different types of cancers with a broad range of mAbs is that it permits selection of the several antigens most suitable as targets for immune attack against each cancer. Expression on normal tissues is, of course, a consideration in this selection, but expression at the secretory border of epithelial tissues does not appear to be a problem (as discussed at greater length in part I of this series; Ref. 1). Antigens expressed at epithelial secretory borders induce neither immunological tolerance nor detectable autoimmunity once antibodies are administered or induced against them. Consequently, if strong expression on $\geq 80\%$ of tumor cells of 60% or more of

Table 4 Protein targets for antibody-mediated immunotherapy^a

Cancer	Antigen ^b
Melanoma	None
Sarcoma	(β hCG)
Neuroblastoma	None
B-cell lymphoma	None
Small cell lung cancer	KSA
Breast	MUC1, MUC5 _{AC} , (KSA), (CEA)
Prostate	MUC2, KSA, (PSMA)
Lung	MUC1, CEA, KSA, (MUC4), (β hCG)
Colon	MUC2, CEA, KSA, (MUC4)
Pancreas	KSA, (MUC4), (CEA), (β hCG)
Gastric	MUC5 _{AC} , CEA, KSA, (MUC3)
Ovarian	MUC1, KSA, (MUC3)
Endometrial	KSA, (MUC1)

^a Targets selected from the 12 antigens tested in this study.

^b Antigens expressed intensely (4+) on $\geq 80\%$ of tumor cells in $\geq 70\%$ of specimens. Antigens in parentheses were expressed on $\geq 80\%$ of tumor cells strongly (3+) on at least 50% or moderately (2+) on at least 60% of specimens.

the cancer specimens tested but not on immune accessible tissues are used as selection criteria, the antigens selected as targets for each cancer are shown in Table 4. The results summarized in Table 4 for protein antigens plus the corresponding tables for ganglioside and carbohydrate antigens in parts I and II of this series (1, 2) provide the basis for selection of multiple antigens as targets for antibody-mediated immune attack against these cancers.

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